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Inhibition of Polyamine Biosynthesis Is a Broad-Spectrum Strategy against RNA Viruses

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ABSTRACT

RNA viruses present an extraordinary threat to human health, given their sudden and unpredictable appearance, the potential for rapid spread among the human population, and their ability to evolve resistance to antiviral therapies. The recent emergence of chikungunya virus, Zika virus, and Ebola virus highlights the struggles to contain outbreaks. A significant hurdle is the availability of antivirals to treat the infected or protect at-risk populations. While several compounds show promise *in vitro* and *in vivo*, these outbreaks underscore the need to accelerate drug discovery. The replication of several viruses has been described to rely on host polyamines, small and abundant positively charged molecules found in the cell. Here, we describe the antiviral effects of two molecules that alter polyamine levels: difluoromethylornithine (DFMO; also called eflornithine), which is a suicide inhibitor of ornithine decarboxylase 1 (ODC1), and diethylnorspermine (DENSpm), an activator of spermidine/spermine N^1-acetyltransferase (SAT1). We show that reducing polyamine levels has a negative effect on diverse RNA viruses, including several viruses involved in recent outbreaks, *in vitro* and *in vivo*. These findings highlight the importance of the polyamine biosynthetic pathway to viral replication, as well as its potential as a target in the development of further antivirals or currently available molecules, such as DFMO.

IMPORTANCE

RNA viruses present a significant hazard to human health, and combatting these viruses requires the exploration of new avenues for targeting viral replication. Polyamines, small positively charged molecules within the cell, have been demonstrated to facilitate infection for a few different viruses. Our study demonstrates that diverse RNA viruses rely on the polyamine pathway for replication and highlights polyamine biosynthesis as a promising drug target.

Polyamines are small, positively charged molecules, derived from arginine, that are involved in several cellular processes in mammalian and nonmammalian cells. Studies carried out on herpesviruses demonstrated that viral capsids contain significant amounts of polyamines that putatively neutralize charges on the viral DNA in order to facilitate compaction and encapsidation (1). Vaccinia virus (2) and bacteriophage R17 (3) also incorporate polyamines into virions. We recently demonstrated that chikungunya virus (CHIKV) and Zika virus (ZIKV), important pathogens responsible for serious outbreaks, rely on polyamines for both translation and transcription (4). The potential role of polyamines in virus replication and the possibility of targeting the polyamine biosynthetic pathway for more diverse array of RNA viruses, including those involved in outbreaks, has not been examined.

Several drugs have been developed that can target the polyamine biosynthetic pathway. Perhaps the best known inhibitor is difluoromethylornithine (DFMO; also called eflornithine), an FDA-approved drug that is used to treat trypanosomiasis (5–7) and hirsutism (8), as well as some cancers (9). DFMO irreversibly inhibits ornithine decarboxylase 1 (ODC1) (Fig. 1A), a critical enzyme in polyamine biosynthesis (10). DFMO is well tolerated in humans, with side effects that are mild and reversible, and can be taken long-term (11). Additionally, DFMO can be administered via several routes, including orally (5), and the compound, dissolved in water and at room temperature, is stable for extended periods of time (12). These characteristics suggest that DFMO may be well suited for treatment of viral infection.

A recently published perspective (13) highlights the importance of developing broad-spectrum antivirals. Ribavirin, a nucleoside analog developed in 1972, is used successfully in the treat-

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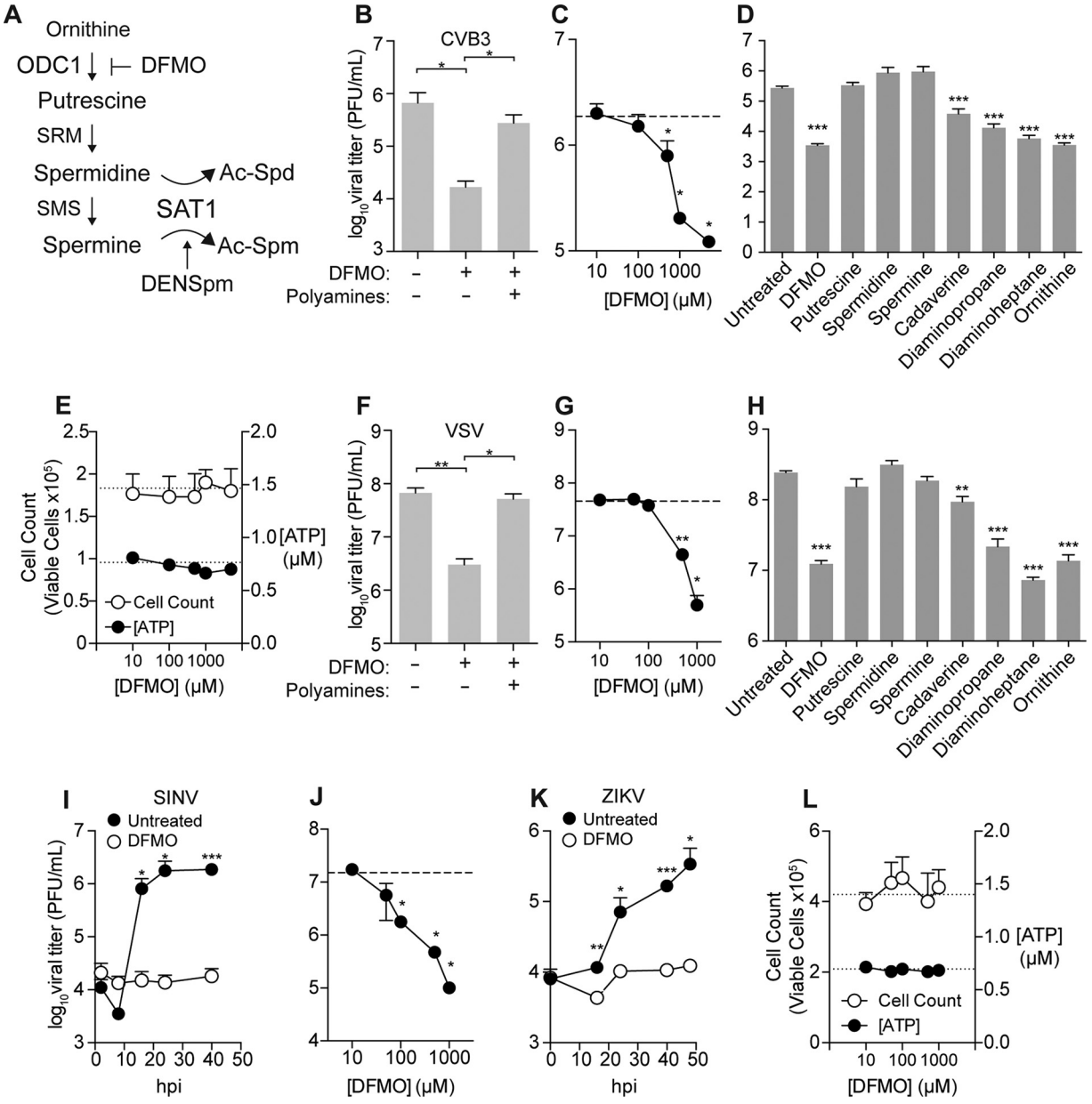

FIG 1 DFMO limits replication of diverse RNA viruses. (A) Diagram presenting the polyamine biosynthetic pathway with pertinent enzymes and inhibitors presented in this study. (B to D) Vero-E6 cells were treated with 500 μM DFMO or different concentrations as indicated for 4 days prior to infection with Coxsackievirus B3 (CVB3) at an MOI of 0.1. At the time of infection, exogenous polyamines were added as indicated. Titers were determined at 24 hpi. (E) Cell counts (open circles, left axis) and viability (closed circles, right axis) were measured on uninfected Vero-E6 cells treated with escalating doses of DFMO for 5 days. Dashed lines indicate levels corresponding to the results for untreated cells. (F to H) BHK-21 cells were treated as described in the legends to panels B to D and subsequently infected with vesicular stomatitis virus (VSV) at an MOI of 0.1 for 16 h. Polyamines were added at the time of infection. (I and J) BHK-21 cells were infected with Sindbis virus (SINV) at the indicated times (I) and treated with the indicated DFMO doses for 24 h (J) at a multiplicity of infection (MOI) of 0.1. (K) Similar to the description in the legend to panel I, cells were infected with Zika virus (ZIKV) at an MOI of 0.1 after pretreatment with 500 μM DFMO in BHK-21 cells, and viral titers determined over the indicated time course. (L) Cell counts and viability were measured on uninfected BHK-21 cells treated with escalating doses of DFMO for 5 days. Statistical significance was determined using Student's *t* test ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent 1 standard error of the mean.

ment of several viral diseases, including hemorrhagic fevers (14). Ribavirin's limited activity against some viruses, including Ebola virus (15), and the development of resistant mutants necessitate investigation into novel pharmaceuticals. Ideally, these drugs would inhibit multiple viruses, possibly by targeting a host pathway that multiple viruses commandeer. The repurposing of previously tested and approved pharmaceuticals would provide significant advantages by reducing the time and cost involved in drug development.

In this study, we discovered that polyamines are important for the replication of a diverse group of viruses. We uncovered the fact that these viruses respond to DFMO in a dose-dependent manner,

as well as to the molecule diethylnorspermine (DENSpm), which acts by upregulating spermidine/spermine N^1-acetyltransferase (SAT1) to deplete polyamines. Further, both work in combination with beta interferon (IFN-β), suggesting that they can be combined with other antiviral molecules. A pretreatment phase with either molecule was required to observe significant reductions in viral titers, though DFMO and DENSpm are able to limit viral infection when added at the time of infection. We further describe the efficacy of DFMO in mouse models of Coxsackievirus B3 (CVB3) and CHIKV infection. Together, these results highlight the importance of the polyamine pathway for diverse viruses, as well as the potential therapeutic use of drugs targeting this pathway.

MATERIALS AND METHODS

Cell culture. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with bovine serum and penicillin-streptomycin. Cells were maintained at 37°C in 5% CO_2.

Drug treatment. DFMO (Tebu Bio and Sigma-Aldrich) was diluted to 50 mM in water; DENSpm (Sigma-Aldrich) was diluted to 1 mM in water. Exogenous polyamines (Sigma-Aldrich) were supplied at 1,000× concentration and used at 5× concentration during treatment. Individual polyamines (Sigma-Aldrich) were diluted to 1 M in water and used at 10 μM. IFN-β (Abcam) was added to cultures 4 h prior to infection at a concentration of 1,000 U/ml.

Five days prior to infection, cells were trypsinized (Life Technologies) and reseeded with medium supplemented with 2% serum. Following overnight attachment, cells were treated with DFMO. DENSpm treatment was done 16 h prior to infection. During infection, medium was cleared from the cells and saved. The same medium was used to replenish the cells following infection. Exogenous polyamines were added immediately following infection where indicated in the figure legends.

Viability assays. Cells were cultured as described above, with escalating concentrations of DFMO and DENSpm or with increasing time of DENSpm treatment. At the described times, cells were assayed for viability using trypan blue exclusion and cell counting. In addition, cells were assayed for ATP levels using CellTiter-Glo reagent (Promega) according to the manufacturer's specifications. A standard curve for ATP concentration was made using 10-fold serial dilutions of ATP, from 1 μM to 100 mM. All measured samples were found to be within the linear range, and [ATP] was calculated from the linear regression equation from the linear portion of the standard curve.

Infection and enumeration of viral titers. Chikungunya virus (CHIKV; La Réunion strain 06-049 [AM258994]), Sindbis virus (SINV; pTR339 strain), and vesicular stomatitis virus (VSV; Indiana strain) were derived from the first passage of virus in BHK-21 cells. CVB3 (Nancy strain) was derived from the first passage in HeLa cells. Dengue virus serotype 1 (DENV1; KDH0026A strain) and Zika virus (ZIKV; HD78788 strain) were derived from the third passage of virus in C6/36 cells. Rift Valley fever virus (RVFV) was derived from patient-isolated strain ZH548. Poliovirus type 1 (PV-1; Sabin type 1 strain) was produced from Hep2C cells. Enterovirus A71 (EV-A71; SEP06 strain) was derived from Vero-E6 cells. Viral stocks were stored at −80°C. For all infections, DFMO and exogenous polyamines were maintained throughout infection as indicated in the figure legends.

For CHIKV, SINV, VSV, and RVFV infection, virus was diluted in serum-free DMEM for a multiplicity of infection (MOI) of 0.1 on BHK-21 cells, unless otherwise indicated. CVB3 infection was performed at an MOI of 0.1 in HeLa cells. The viral inoculum was overlaid on the cells for 30 min to 1 h; virus was then cleared from the cells and the cells were washed three times with phosphate-buffered saline (PBS). The medium was replenished as described above.

Supernatants were collected from CHIKV, SINV, RVFV, and VSV infections at 24 h postinfection (hpi) unless otherwise indicated. CVB3 supernatant and infected cells were freeze-thawed twice at 24 hpi and scraped prior to titration. Dilutions of cell supernatants were prepared in serum-free DMEM and used to inoculate confluent monolayers of Vero-E6 cells for 30 min to 1 h at 37°C. The cells were then overlaid with 0.8% agarose in DMEM containing 1.6% newborn calf serum (NBCS). After 72 h of incubation, cells were fixed with 4% formalin and revealed with crystal violet solution (10% crystal violet [Sigma-Aldrich], 20% ethanol). Plaques were enumerated and used to back calculate the number of PFU per milliliter of collected volume. PV and EV-A71 infections were performed as described above in HeLa cells at an MOI of 0.1. The cell cultures were frozen, and the supernatants used to determine viral titers via 50% tissue culture infective dose ($TCID_{50}$).

Yellow fever virus (YFV) infection was performed as described above in BHK-21 cells at an MOI of 0.01. Supernatants were collected at 72 hpi, and the titers determined by focus-forming assay with a 1.5% carboxymethylcellulose overlay and 96-h incubation. Foci were revealed with an anti-nonstructural protein 1 (NS1)-specific antibody for enumeration.

The infectious clone of Middle East respiratory syndrome coronavirus (MERS-CoV) was recovered as described previously (16). Viral stocks were maintained at −80°C. For MERS-CoV infection, medium was removed from Vero CCL-81 cells and replaced with viral inoculum. Cells were infected at an MOI of 0.1 in complete DMEM with or without DFMO and/or 5× polyamines. Viral supernatant was harvested at 48 hpi.

MERS-CoV titers were determined by plaque assay on Vero CCL-81 cells. Briefly, 10-fold dilutions were prepared in gel saline (PBS [+Mg/+Ca] containing 0.3% [wt/vol] gelatin) and used to inoculate Vero CCL-81 cells for 45 min at 37°C. Cells were then overlaid with DMEM containing 1% agarose. After 72 h of incubation, plaques were enumerated and used to calculate the number of PFU/ml.

For DENV and ZIKV, dilutions were prepared in serum-free Leibovitz's L-15 (Life Technologies) and used to inoculate C6/36 cells at an MOI of 1 for 2 h at 28°C. Cells were then overlaid with 1.6% carboxymethylcellulose in L-15 medium containing 5% fetal calf serum. After 5 days of incubation, cells were fixed with 4% paraformaldehyde. Cells were stained by mouse pan-flavivirus anti-E antibody (4G2; RD-Biotech) and foci revealed by goat anti-mouse antibody coupled to fluorescein isothiocyanate (FITC; Sigma). Foci were enumerated and used to back calculate the number of focus-forming units (FFU) per milliliter of collected volume.

Japanese encephalitis virus (JEV) was obtained from transfection of HEK293T cells with the RP-9 infectious clone (17), followed by expansion in C6/36 cells. For titration of JEV and West Nile virus (WNV), the BHK-21 cells were overlaid with DMEM supplemented with 1.6% carboxymethylcellulose, 10 mM HEPES buffer, 72 mM sodium bicarbonate, and 2% fetal bovine serum and incubated at 37°C for 32 h for JEV and 48 h for WNV. The carboxymethylcellulose overlay was aspirated, and the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% Triton X-100 for 5 min. After fixation, the cells were washed with PBS and incubated for 1 h at room temperature with anti-E antibody (4G2), followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody. The assays were developed with the Vector VIP peroxidase substrate kit (catalog no. SK-4600; Vector Laboratories) according to the manufacturer's instructions.

Meninges-free cortices from C57BL/6J mouse embryos (gestational day 17) were dissected in Hanks balanced salt solution (HBSS; Life Technologies) and treated for 10 min with Liberase High dispase (0.52 Wünsch units/ml; Roche) and DNase (400 units/ml; Roche) before titration. Enzymes were neutralized with fetal calf serum, and dissociated cells rinsed two times in HBSS. Pelleted cells were then resuspended in neurobasal medium supplemented with 2% B-27 supplement, 1% N-2 supplement, 25 μM β-mercaptoethanol, 2 mM GlutaMax, 10 mM HEPES, and 50 μg/ml gentamicin. Cells were plated (3×10^4 cells/cm^2) in black 96-well CellBind plates (Corning) previously coated with 50-μg/ml poly-D-lysine (product no. P6407; Sigma), followed by 5 μg/ml Laminine (Sigma). Cells

were maintained at 37°C in 9% CO_2. On day 1, cultures were treated with 25-μg/ml uridine and 10-g/ml fluorodeoxyuridine (to eliminate proliferating glial cells). DFMO, combined or not with polyamines, was added on day 1. On day 4, neurons were exposed to rabies virus (RABV) (MOI of 0.001), and they were fixed 3 days later for immunofluorescent labeling with anti-RABV nucleocapsid antibody. The plates were scanned with an ArrayScan reader, and nucleocapsid-positive inclusions were automatically quantified in at least 1,500 neurons per condition with the Cellomics NeuroProfiler BioApplication software (Thermo Fisher Scientific).

Calculation of the effect of DFMO was done by dividing the titers obtained without DFMO treatment by the titers obtained with DFMO treatment.

TLC determination of polyamines. Polyamines were separated by thin-layer chromatography (TLC) as previously described (18). Cells were treated as described. At the time of analysis, the cells were trypsinized and centrifuged. The pellets were washed with PBS and then resuspended in 250 μl of 2% perchloric acid. Samples were then incubated overnight at 4°C. Samples were centrifuged for 30 m at 13,000 rpm, and supernatants collected. Two hundred microliters of supernatant was combined with 400 μl of 5-mg/ml dansyl chloride (Sigma-Aldrich) in acetone and 200 μl of saturated sodium bicarbonate. Samples were incubated in the dark overnight at room temperature. Excess dansyl chloride was cleared by incubating the reaction mixture with 100 μl of 150-mg/ml proline (Sigma-Aldrich). Dansylated polyamines were extracted with 100 μl of toluene (Sigma-Aldrich). Five microliters of each sample was added in small spots to the TLC plate (silica gel matrix; Sigma-Aldrich) and exposed to ascending chromatography with 2:3 cyclohexame/ethylacetate. The plate was dried and visualized via exposure to UV.

Amplification of CHIKV clinical isolates. A serum sample obtained from a patient during the 2013 Caribbean chikungunya virus outbreak was added to C6/36 cells and passaged twice to generate a viral stock. The titer of the viral stock was determined as described above.

Plaque reduction assay. BHK-21 or Vero-E6 cells were split into 6-well dishes 1 day prior to infection. Tenfold serial dilutions of virus were prepared and used to infect the monolayer. Immediately after the inoculum was added to the cells, 1 mM DFMO with or without 2× polyamines was added to the medium. The cells were allowed to incubate at 37°C for 1 h and were then overlaid with medium-agarose solution. VSV plaques were developed at 2 dpi, and ZIKV plaques at 4 dpi. For all other viruses, plaques developed over the course of 3 days, at which point they were revealed with crystal violet solution as described above. The plates were scanned, and ImageJ was used to measure plaque radii and areas.

Mouse treatment and infection. Mice were kept in the Pasteur Institute animal facilities under biosafety level 2 conditions, with water and food supplied *ad libitum*, and they were handled in accordance with institutional guidelines for animal welfare. For DFMO treatment, DFMO was added to the drinking water at a concentration of 1% (1 g/100 ml) and mice were maintained with this drinking water for 7 days prior to infection, as well as during the 3 days of infection. For CVB3, BALB/c mice were infected intraperitoneally with 2,000 $TCID_{50}$ in 0.25 ml. At 3 days postinfection (dpi), organs were harvested and weighed, and a Precellys 24 homogenizer (Bertin Technologies, Bretonneux, France) was used to grind tissues. For CHIKV, C57BL/6 mice were injected subcutaneously with 10^5 PFU in the hind leg near the ankle, and tissues were harvested at 24 hpi. Viral titers were enumerated as described above and expressed as PFU per mg of tissue, normalized to the sample mass.

Ethics statement. Mouse studies were performed in accordance with international guidelines for animal research (35), and all experiments were performed in the appropriate containment facility. The protocols were approved by the Institutional Committees on Animal Welfare of the Pasteur Institute (OLAW assurance number A5476-01). All efforts were made to minimize suffering.

Statistical analysis. Prism 6 (GraphPad, La Jolla, CA) was used to generate graphs and perform statistical analysis. For all analyses, one-tailed Student's *t* test was used to compare groups, with an α level of 0.05.

RESULTS

DFMO limits the replication of diverse RNA viruses. To determine the diversity of viruses that are impacted by polyamine depletion, we examined whether DFMO pretreatment of cells could limit the replication of the positive-stranded enteroviruses and negative-stranded rhabdoviruses, using Coxsackievirus B3 (CVB3) and vesicular stomatitis virus (VSV) as representatives from their respective viral lineages. We pretreated Vero-E6 cells with 500 μM DFMO for 4 days prior to infection with CVB3 at an MOI of 0.1 At the time of infection, polyamines were replenished via a polyamine mixture, as indicated in the figure. At 24 hpi, we measured titers by plaque assay and found a significant reduction in viral titers in DFMO-treated cells compared to the titers in untreated cells (Fig. 1B). As we had observed for chikungunya virus (CHIKV) previously (4), exogenous polyamine addition rescued the viral titers to nearly the levels in untreated cells. In a similar vein, Vero-E6 cells were treated with escalating doses of DFMO, from 10 to 5,000 μM, and infected as described above. Again, we observed a dose-response inhibition of CVB3 replication (Fig. 1C). Finally, we determined whether biogenic or synthetic polyamines could rescue CVB3 titers, so after the pretreatment with 500 μM DFMO, cells were replenished with single polyamines. As we had observed for CHIKV (4), only putrescine, spermidine, and spermine were able to fully rescue viral titers (Fig. 1D), suggesting that these viruses rely on very specific forms of polyamines for their replication. The cells exhibited no signs of toxicity, as measured by viable cell count (Fig. 1E, open circles) or ATP content (Fig. 1E, closed circles), as we reported previously (4).

Similarly, BHK-21 cells were treated with DFMO and infected with VSV for 16 h. Similar to what we had observed with CVB3 (Fig. 1B to D), we found that VSV was sensitive to DFMO and could be rescued with exogenous polyamines (Fig. 1F), that this sensitivity was dose dependent (Fig. 1G), and that the biogenic polyamines were able to rescue viral titers (Fig. 1H). Together, these results implicate polyamines in the replication of diverse viruses.

We had previously observed that CHIKV and Zika virus (ZIKV) were sensitive to polyamine depletion (4), and to expand on these data, we used a related virus, Sindbis virus, which is a close relative to CHIKV but was shown in a previous study to not be affected by DFMO treatment (19). BHK-21 cells were pretreated with 500 μM DFMO and infected at an MOI of 0.1, and the titers measured over a time course of infection (Fig. 1I). Similar to CHIKV, we observed a significant reduction in viral titers with DFMO treatment. Also similar to CHIKV, we observed a dose-dependent reduction in viral titers with escalating doses of DFMO after 24 h of infection (Fig. 1J). The same phenotype was observed for ZIKV (Fig. 1K), confirming our prior result that ZIKV is sensitive to polyamines. As with Vero-E6 cells, we observed no toxicity from DFMO treatment (Fig. 1L).

To expand on these observations, we tested the antiviral activity of DFMO against a broad range of RNA viruses. All viruses were found to be sensitive; the viruses tested were Middle East respiratory syndrome coronavirus (MERS-coronavirus), flaviviruses dengue virus serotype 1 (DENV1), Japanese encephalitis virus (JEV), and yellow fever virus (YFV), enteroviruses enterovirus A71 (EV-A71) and poliovirus (PV), bunyavirus Rift Valley fever virus (RVFV), and rhabdovirus rabies virus (RABV). These rep-

TABLE 1 Properties of viruses used in this study and the effects of DFMO

Virus	Family	Genus	Sense	Envelope	Segmented	Effect of DFMO (fold reduction in titer)[a]	Cell type
Chikungunya virus[b]	*Togaviridae*	*Alphavirus*	+	Yes	No	200**	BHK-21
						8*	Murine fibroblasts
						12**	Vero
						12**	Human fibroblasts
						28*	C6/36
Sindbis virus	*Togaviridae*	*Alphavirus*	+	Yes	No	10***	BHK-21
MERS coronavirus	*Coronaviridae*	*Coronavirus*	+	Yes	No	30***	Vero
Dengue virus, serotype 1	*Flaviviridae*	*Flavivirus*	+	Yes	No	60***	BHK-21
Zika virus[b]	*Flaviviridae*	*Flavivirus*	+	Yes	No	15***	BHK-21
Japanese encephalitis virus	*Flaviviridae*	*Flavivirus*	+	Yes	No	5**	BHK-21
Yellow fever virus	*Flaviviridae*	*Flavivirus*	+	Yes	No	90*	BHK-21
Enterovirus A71	*Picornaviridae*	*Enterovirus*	+	No	No	12*	HeLa
Coxsackievirus B3	*Picornaviridae*	*Enterovirus*	+	No	No	10*	HeLa
Poliovirus	*Picornaviridae*	*Enterovirus*	+	No	No	20*	HeLa
Rift Valley fever virus	*Bunyaviridae*	*Phlebovirus*	−	Yes	Yes	200***	BHK-21
Vesicular stomatitis virus	*Rhabdoviridae*	*Vesiculovirus*	−	Yes	No	20**	BHK-21
Rabies virus	*Rhabdoviridae*	*Lyssavirus*	−	Yes	No	2***	Cortical neurons

[a] Calculated by dividing titers from untreated cells by titers from DFMO-treated cells. Statistical significance was determined using a one-tailed Student's *t* test to compare the results for untreated and DFMO-treated cultures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[b] As described previously (4).

resent positive- and negative-stranded viruses, enveloped and nonenveloped viruses, and segmented and nonsegmented viruses (summarized in Table 1). In all cases, viral titers were rescued with exogenous polyamine treatment (Fig. 2). Thus, it appears that several RNA viruses rely on polyamines for their replication.

DENSpm inhibits RNA virus replication. Another mechanism to deplete cells of polyamines is through the activation of SAT1 via DENSpm, which works to upregulate SAT1 activity within the cell. We had previously observed that both CHIKV and ZIKV are sensitive to SAT1 overexpression and that ZIKV titers are sensitive to DENSpm in a dose-dependent manner (4). To determine whether this mechanism of polyamine depletion was also effective against other RNA viruses, we examined whether these viruses were sensitive to DENSpm treatment.

Vero-E6 cells were treated with DENSpm 16 h prior to infection with CVB3 at an MOI of 0.1 and with VSV at an MOI of 0.01. Over a time course of infection, the viral titers were determined via plaque assay. For both viruses, we observed a significant reduction in viral titers at all time points examined (Fig. 2A and B), suggesting that these viruses are also sensitive to DENSpm. We confirmed that these viruses are sensitive in a dose-dependent manner (Fig. 2C and D), as is CHIKV (Fig. 2E), suggesting that DENSpm restricts the viral replication of diverse RNA viruses. Similar to treatment with DFMO, DENSpm did not affect cellular viability in these assays (Fig. 3F). To confirm that DENSpm was affecting SAT1 expression and polyamine levels within the cell, we performed Western blot analysis for SAT1 and thin-layer chromatography for polyamines and observed increases in SAT1 expression and alterations in polyamine levels concomitant with changes in viral titers (Fig. 3G).

Polyamine depletion blocks viral replication in combination with IFN-β. Combination therapy in the treatment of viral infection is a common feature to target viral replication at multiple stages, as well as to reduce the likelihood of the development of resistance. To determine whether polyamine depletion could be combined with another known antiviral molecule, we examined the effect of cotreating cells with DFMO and beta interferon, a potent antiviral molecule. When we treated Vero-E6 cells with 500 μM DFMO for 4 days and 1,000-U/ml IFN-β for 4 h prior to infection with CVB3 (Fig. 4A), VSV (Fig. 4B), and CHIKV (Fig. 4C), we observed that both molecules were able to reduce viral replication to differing degrees. When the molecules were combined, CHIKV titers were synergistically reduced, suggesting that DFMO combined with IFN-β may be a successful treatment for select viruses.

Similarly, Vero-E6 cells were treated with 10 μM DENSpm for 16 h and 1,000 U IFN-β for 4 h prior to infection with the same viruses. Similar to the results for DFMO, we saw reductions in titers for each drug alone, but the combination was not uniformly synergistic (Fig. 4D to G). Together, these results suggest that the combination of IFN-β, a commonly administered antiviral, with either polyamine-depleting agent may not significantly affect viral replication compared to single treatment.

Polyamine depletion limits viral replication in a time-dependent manner. Treatment of a viral infection prior to exposure is not feasible in many situations, and most viral infections are not detected until long after viral infection has been established. SAT1 rapidly depletes polyamines from cells (20), and thus, DENSpm, which upregulates SAT1, may serve to limit the spread of viral infection more effectively than DFMO-mediated polyamine depletion. To investigate this possibility, we treated Vero-E6 cells with 10 μM DENSpm at various times prior to and after infection. At 24 hpi, we observed that CVB3 titers were reduced when DENSpm was added as late as 4 hpi (Fig. 5A). However, the greatest effect on viral replication was achieved when cells were pretreated with DENSpm 4 to 16 h prior to infection. Interestingly, VSV titers were strongly dependent on this pretreatment (Fig. 5B), as even treatment with DENSpm at the time of infection failed to affect viral titers, possibly due to the fast replication kinetics of this virus. With CHIKV, we observed that viral titers were affected similarly to CVB3: treatment as late as 4 hpi was effective, but pretreatment was more effective (Fig. 5C). Additionally, we

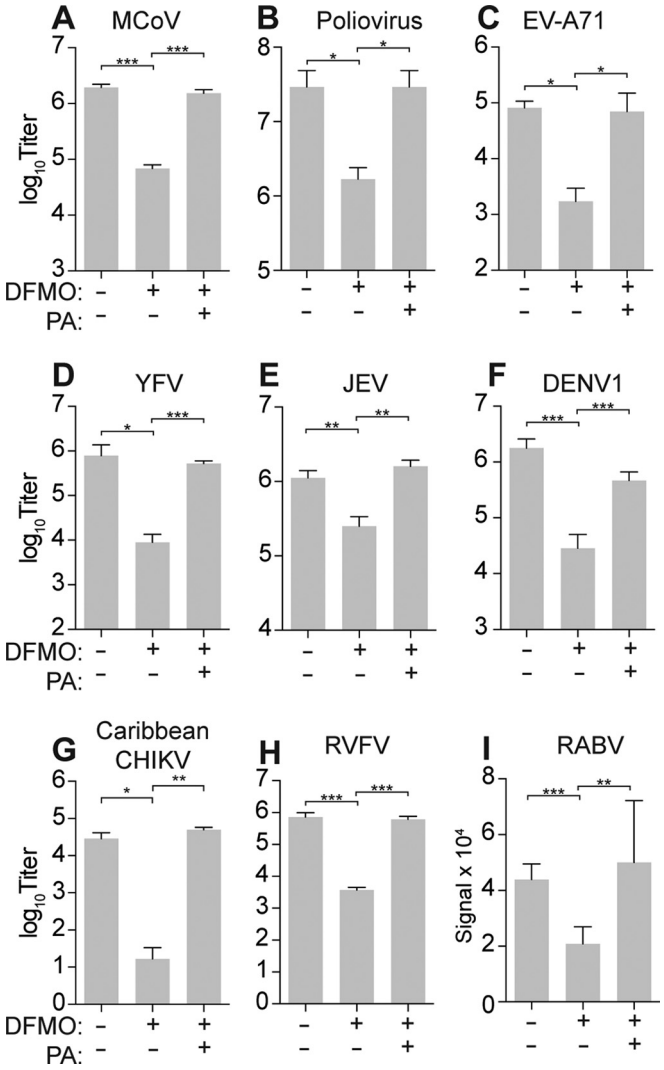

FIG 2 Polyamine depletion restricts the replication of diverse families of viruses. Virus titers were determined following a 4-day 500 μM DFMO treatment and rescue with exogenous polyamines, added at the time of infection. (A) Middle East respiratory syndrome coronavirus (MERS-CoV [MCoV]) at 24 hpi in Vero81 cells; (B) poliovirus (PV) at 24 hpi in HeLa cells; (C) Enterovirus A71 (EV-A71) at 24 hpi in HeLa cells; (D) Yellow fever virus (YFV) at 96 hpi in BHK-21; (E) Japanese encephalitis virus (JEV) at 24 hpi in BHK-21 cells; (F) Dengue virus-1 (DENV1) at 48 hpi in BHK-21 cells; (G) Caribbean isolate of chikungunya virus (CHIKV) at 24 hpi in BHK-21 cells; (H) Rift Valley fever virus (RVFV) at 24 hpi in BHK-21 cells; (I) rabies virus (RABV) at 24 hpi in primary cortical neurons. MERS-CoV, CVB3, JEV, WNV, VSV, and RVFV titers were measured by plaque assay; PV and EV-A71 titers were measured by $TCID_{50}$; DENV1 and YFV titers were determined by fluorescent focus assay; and RABV infection was determined by quantitation of the signal intensity of somatic inclusions. Statistical significance versus the results for the untreated control was determined using one-tailed Student's *t* test; $n = 3$ replicates for all viruses except DENV ($n = 8$), ZIKV ($n = 9$), MERS ($n = 6$), and RABV ($n = 6$). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Bars and error bars represent mean results ± 1 standard deviation.

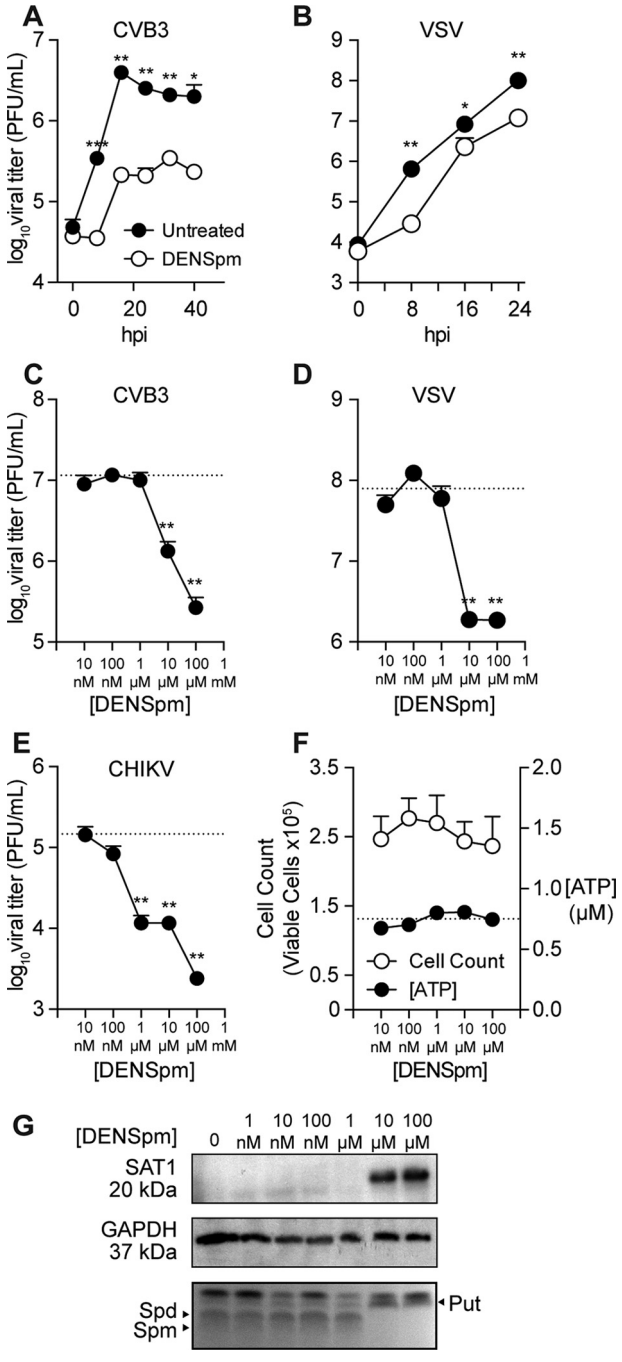

FIG 3 DENSpm inhibits RNA virus replication. (A and B) Time course of infection of Vero-E6 cells treated with 10 μM DENSpm for 16 h prior to infection with CVB3 (A) or VSV (B) at an MOI of 0.1 or 0.01, respectively. Titers were determined at the times indicated. (C to E) Vero-E6 cells were pretreated with escalating doses of DENSpm for 16 h prior to infection with CVB3 at an MOI of 0.1 (C), VSV at an MOI of 0.01 (D), or CHIKV at an MOI of 0.1 (E). Titers were determined at 24 hpi (40 h posttreatment). (F) Cell counts (open circles, left axis) and viability (closed circles, right axis) were measured for uninfected Vero-E6 cells treated with escalating doses of DENSpm for 40 h. Dashed lines indicate levels corresponding to the results for untreated cells. (G) Western blot analysis of SAT1 and GAPDH (top two panels) and thin-layer chromatographic analysis of polyamines (bottom panel; polyamines labeled for spermidine [Spd], spermine [Spm], and putrescine [Put]) were performed on uninfected samples run, measured at 40 h posttreatment. Statistical significance was determined using Student's *t* test ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent 1 standard error of the mean.

found that over a time course of DENSpm treatment similar to the times presented in Fig. 5A to C, no changes in cell viability were observed (Fig. 5D). These results suggest that, in order to reduce viral titers, pretreatment with DENSpm is necessary for most but not all viruses.

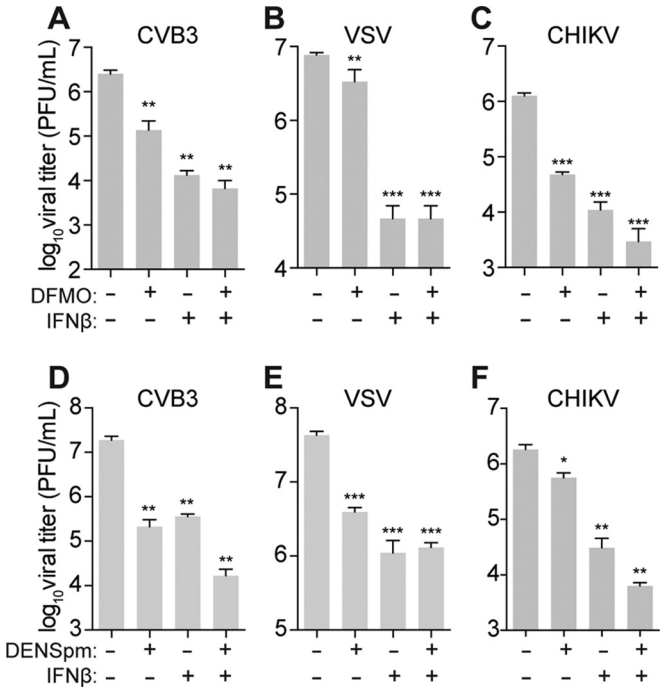

FIG 4 Polyamine depletion blocks RNA virus replication in combination with IFN-β. (A to C) BHK-21 cells were not treated or pretreated with 500 μM DFMO for 4 days and with 1,000 U IFN-β for 4 h, as indicated, prior to infection with CVB3 at an MOI of 0.1 (A), VSV at an MOI of 0.01 (B), and CHIKV at an MOI of 0.1 (C). Titers were determined at 24 hpi. (D to F) Vero-E6 cells were not treated or treated with 10 μM DENSpm for 16 h and 1,000 U IFN-β for 4 h, as indicated, prior to infection with CVB3 (D), VSV (E), and CHIKV (F). Statistical significance was determined using Student's *t* test ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent 1 standard error of the mean.

Previously, we had observed that at least a 2-day pretreatment with DFMO was necessary to significantly reduce CHIKV titers (4). To determine whether we could reduce viral replication with DFMO without pretreatment, we used a plaque size reduction assay. Confluent monolayers of Vero-E6 or BHK-21 cells were infected with 10 to 20 PFU, and 1 mM DFMO was added to the inoculum with or without exogenous polyamines. With this assay, the virus is able to replicate within infected cells and the effectiveness of polyamine depletion on viral spread can be determined by the size of the plaques: smaller plaques indicate that the viruses were unable to spread through the monolayer. In all cases, DFMO treatment at the time of infection reduced viral spread, as measured by plaque area (Fig. 5E to J). Additionally, the supplementation of these monolayers with polyamines rescued this phenotype.

Polyamine depletion limits viral replication in mammalian models. We had previously demonstrated that the treatment of several model organisms with DFMO can limit SINV infection, specifically in the *Drosophila* and zebrafish systems (4). To determine whether DFMO can be effective in a mammalian model, we provided BALB/c mice with 1% DFMO in their drinking water so that the drug was available *ad libitum*. After 7 days of this treatment, the mice were infected with 1,000 $TCID_{50}$ of CVB3 intraperitoneally. After 3 days, organs were harvested and homogenized, and the viral titers were determined via plaque assay. In the primary target organs of the spleen, pancreas, and heart, we observe slight, statistically insignificant changes in viral titers, with at most a threefold reduction in viral titers (Fig. 6A to C). However, in the lung, liver, and kidneys, we saw significant differences, with four-, seven-, and threefold reductions in titer, respectively (Fig. 6D to F). These results correlate well with published literature describing higher levels of DFMO in the liver and kidneys and less DFMO present in the spleen, pancreas, and heart (21). Overall, we saw a trend toward decreasing titers in all organs, but the differences were not as striking as the *in vitro* data.

Similar to our CVB3 infections, we provided C57BL/6 mice with 1% DFMO in the drinking water for 7 days prior to footpad injection with 10^5 PFU CHIKV. At 24 hpi, tissues were collected and homogenized, and the viral titers were determined via plaque assay. We observed the same trend as with CVB3: the titers in the tissues analyzed tended to be lower in DFMO-treated mice than in the controls (Fig. 6G to I), though the differences were not as striking as in our *in vitro* model. As a whole, these results suggest that polyamine depletion *in vivo* has the potential to reduce viral replication; however, further optimization at the level of treatment regimen, as well as drug design, could provide enhanced antiviral activity.

DISCUSSION

Although some DNA and RNA viruses have been described to rely on polyamines for replication, the diversity of these viruses has not been appreciated. We previously described our findings that flaviviruses and alphaviruses require polyamines, specifically at the levels of translation and transcription (4), and we now expand these observations to viruses from diverse families and reveal that this pathway has potential for therapeutic intervention. Indeed, we show that by targeting the polyamine pathway either through DFMO or DENSpm, we can significantly reduce RNA virus replication. Because polyamines are ubiquitous and abundant molecules, present at concentrations up to 1 mM (22), the observation that viruses rely on this pathway is fitting: these viruses have evolved in the presence of polyamines and have likely evolved mechanisms to utilize them for replication.

Polyamines play many roles within the cell, including binding and altering the conformation of RNA, modifying transcription and translation rates, and affecting signaling pathways (23). Viruses likely uncovered mechanisms to use polyamines in a similar manner. Because such a diverse group of viruses relies on polyamines for replication, it is possible that a more general function of polyamines may be at work for these viruses. For instance, polyamines are important in RNA structure (24), which is crucial for many viruses. Additionally, polyamines are important for cellular translation via the hypusination of eIF5A (25). Our prior results with CHIKV and DENV suggest that polyamines are required for the translation of the viral genome (4), supporting this hypothesis.

Combating infectious diseases will perpetually present significant challenges due to their sporadic emergence and proclivity to evolve resistance. The development of novel antivirals remains a priority for fighting disease, but the repurposing of previously approved pharmaceuticals can significantly accelerate the process of making antivirals available (26). Recent work highlights the fact that currently available pharmaceuticals show promise against several viruses, including CHIKV(27), MERS-CoV (28), and Ebola virus (29). As we have described here, the polyamine biosynthesis inhibitor DFMO shows significant promise against sev-

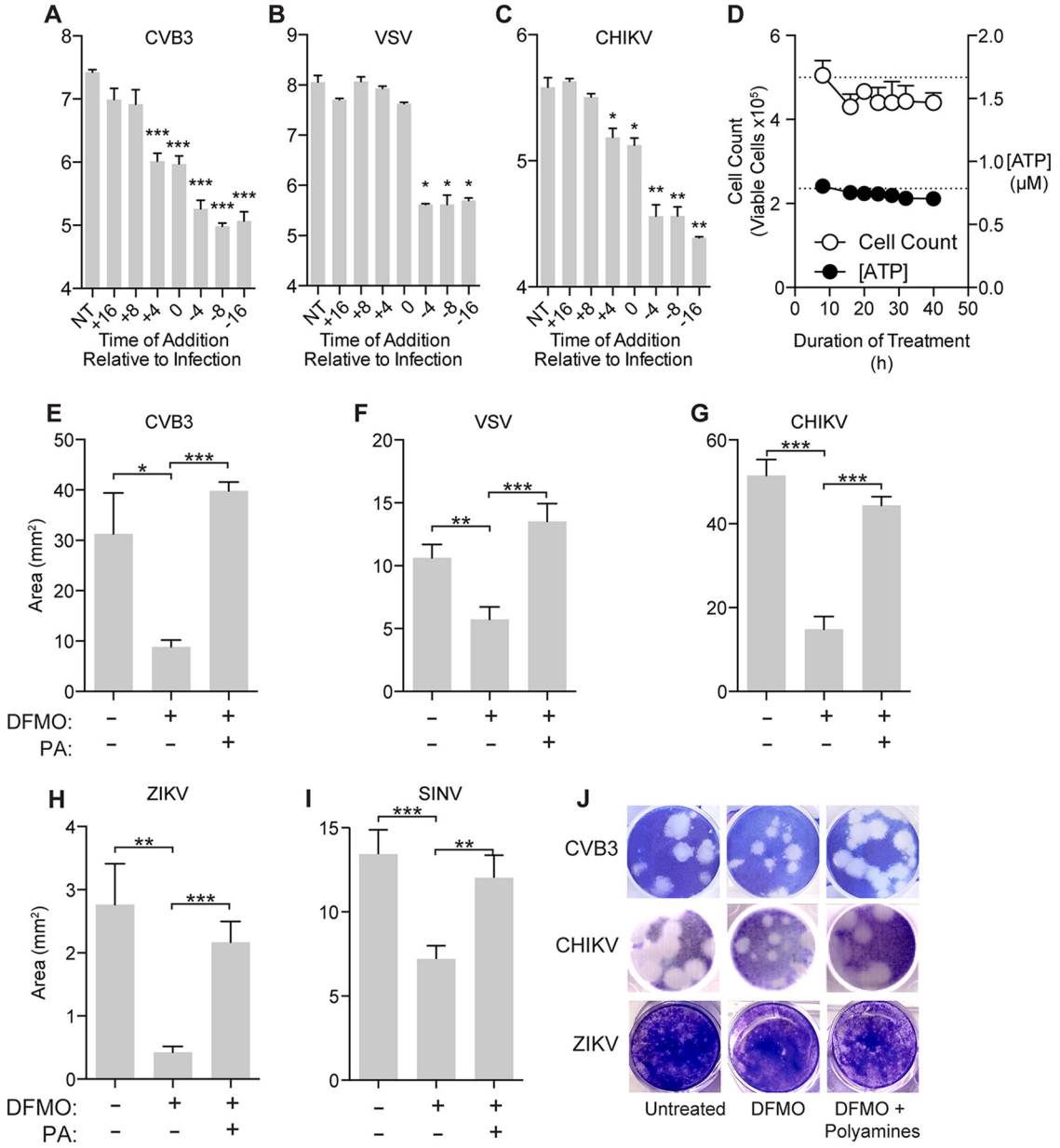

FIG 5 Polyamine depletion limits viral replication in a time-dependent manner. BHK-21 cells were treated with 10 μM DENSpm at the indicated times prior to and after infection with CVB3 at an MOI of 0.1 (A), VSV at an MOI of 0.01 (B), and CHIKV at an MOI of 0.1 (C) for 24 h. (D) Cell counts (open circles, left axis) and viability (closed circles, right axis) were measured on uninfected Vero-E6 cells treated with DENSpm for different times as described in the legend to panels A to C. Dashed lines indicate levels corresponding to the results for untreated cells. (E to I) Confluent monolayers of cells were treated with DFMO and a polyamine mixture (PA) at the time of infection with CVB3 (E), VSV (F), CHIKV (G), ZIKV (H), and SINV (I) in a plaque-reduction assay. Plaques were developed after 4 days (H), 3 days (E, G, and I), or 2 days (F). (J) Representative plaques from CVB3, CHIKV, and ZIKV, treated as indicated. Statistical significance was determined using Student's *t* test ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent 1 standard error of the mean.

eral devastating viruses, including those involved in recent outbreaks. Given that DFMO is well tolerated in humans and has mild, reversible side effects, DFMO may prove useful in combating viral infection. We observed that DFMO is most effective with pretreatment (4), which serves to reduce polyamine content prior to viral infection. Treating large populations with DFMO prophylactically is not economical, though it may hold promise in outbreak situations. Specifically, the treatment of health care workers or other at-risk populations may stem the spread of outbreak viruses. However, our results also suggest that DFMO is effective after exposure to a low infectious dose of virus. More effective derivatives of DFMO or the combination of DFMO with other therapeutics may serve to enhance its antiviral activity.

DFMO is a stable molecule (12), dissolves in water, and is bioavailable in several forms (30). Its stability lends itself well to transport globally, including to regions were refrigeration is not readily available. Sanofi, a manufacturer of DFMO, has pledged support for its production for the treatment of trypanosomiasis (31), and the additional antiviral properties of DFMO emphasize the importance of making this drug available. Future modification

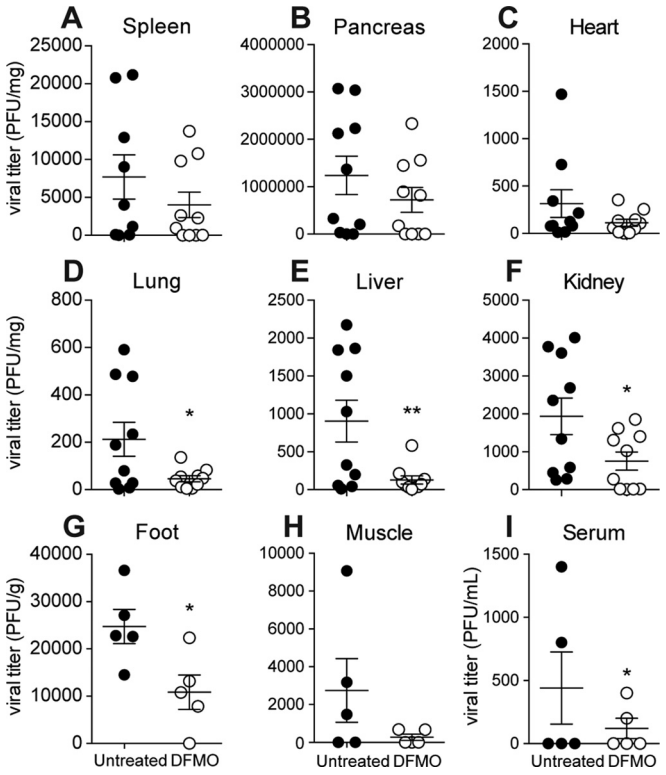

FIG 6 Polyamine depletion limits viral replication *in vivo*. (A to F) Mice were provided 1% DFMO in drinking water *ad libitum* for 7 days prior to infection with 1,000 $TCID_{50}$ of CVB3 intraperitoneally. At 3 dpi, viral titers in the spleen (A), pancreas (B), heart (C), lung (D), liver (E), and kidney (F) were determined by plaque assay. (G to I) As described in the legend to panels A to F, mice were provided DFMO for 7 days prior to infection by footpad injection of 10^5 PFU of CHIKV. At 24 hpi, organs were harvested and titers in the foot (G), muscle (H), and serum (I) were determined by plaque assay. Statistical significance was determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$. Symbols show the results for individual mice, horizontal lines represent the mean values for the groups, and error bars represent 1 standard error of the mean.

and optimization of DFMO may lead to the development of safer and more effective formulations that will render prophylactic and postexposure treatments more feasible. Furthermore, combining DFMO with other well-established antivirals may also prove important in combating viral disease.

Our *in vivo* results in mice, as well as our prior results in zebrafish and *Drosophila melanogaster* (4), show that polyamines are required for optimal viral replication in multiple hosts. Given DFMO's effectiveness in model organisms, the drug may hold promise in humans. However, treating humans with DFMO may require a prolonged prophylactic phase in order to significantly reduce polyamines. Polyamines are found in the human diet (32), and thus, more hurdles may exist in reducing polyamine content within the human body than under the controlled laboratory conditions. Despite the modest, yet significant, reductions in CVB3 and CHIKV replication, DFMO still showed efficacy in the mouse model. We measured viral titers at the peak of viral infection, leaving open the possibility that other parameters of infection, such as morbidity or mortality, may be improved with polyamine depletion, an important area of future investigation. Additionally, modification of DFMO, exploration of other polyamine-targeting therapies, and combination with other known antivirals may provide improved efficacy *in vivo*. In this system, we were unable to control the precise dose of DFMO that the mice received due to the form of administration (DFMO in drinking water provided *ad libitum*); however, in a clinical setting where we can control dosing, through intravenous administration, for example, we may better be able to improve the efficacy of DFMO and restrict viral replication further *in vivo*. The positive correlation between titer and disease severity, including for CHIKV (33) and dengue virus (34), suggests that these decreases in viral titers *in vivo* may be sufficient to reduce disease burden. Additionally, reducing viral titers in the short-term may provide the host time to develop an adaptive immune response to better combat viral infection. Thus, the polyamine biosynthetic pathway represents a promising drug target.

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